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The Anti-Microbial Efficacy of Plant Essential Oil Combinations and Interactions with Food Ingredients

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Title: "**The anti-microbial efficacy of plant essential oil combinations and interactions with food ingredients**"

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Running Title: Antimicrobial efficacy of plant EOs and interactions with food ingredients

Abstract

The objective of this study was to evaluate the efficacy of plant essential oils (EOs) in combination and to investigate the effect of food ingredients on their efficacy. The EOs assessed in combination included basil, lemon balm, marjoram, oregano, rosemary, sage and thyme. Combinations of EOs were initially screened against *B. cereus*, *E. coli*, *L. monocytogenes* and *P. aeruginosa* using the spot-on-agar test. The influence of varying concentrations of EO combinations on efficacy was also monitored using *E. coli*. These preliminary studies showed promising results for oregano in combination with basil, thyme or marjoram. The checkerboard method was then used to quantify the efficacy of oregano, marjoram or thyme in combination with the remainder of selected EOs. Fractional inhibitory concentrations (FIC) were calculated and interpreted as synergy, addition, indifference or antagonism. All the oregano combinations showed additive efficacy against *B. cereus*, and oregano combined with marjoram, thyme or basil also had an additive effect against *E. coli* and *P. aeruginosa*. The mixtures of marjoram or thyme also displayed additive effects in combination with basil, rosemary or sage against *L. monocytogenes*. The effect of food ingredients and pH on the antimicrobial efficacy of oregano and thyme was assessed by monitoring the lag phase and the maximum specific growth rate of *L. monocytogenes* grown in model media. The model media included potato starch (0, 1, 5 or 10%), beef extract (1.5, 3, 6 or 12%), sunflower oil (0, 1, 5 or 10%) and TSB at pH levels of 4, 5, 6 or 7. The antimicrobial efficacy of EOs was found to be a function of ingredient manipulation. Starch and oils concentrations of 5% and 10% had a negative impact on the EO efficacy. On the contrary, the EOs were more effective at high concentrations of protein, and at pH 5, by comparison with pH 6 or 7. This study suggests that combinations of EOs could minimize application concentrations and consequently reduce any adverse sensory impact in food. However, their application for microbial control might be affected by food composition, therefore, careful selection of EOs appropriate to the sensory and compositional status of the food system is required. This work shows that EOs might be more effective against food-borne pathogens and spoilage bacteria when applied to ready to use foods containing a high protein level at acidic pH, as well as lower levels of fats or carbohydrates.

Key words**: essential oils, antimicrobial, synergy, food ingredients, food application**

1. Introduction

Essential oils (EOs) are aromatic and volatile oily liquids obtained from plant material. They are normally formed in special cells or groups of cells, found in leaves and stems, and commonly concentrated in one particular region such as leaves, bark or fruit (Oussalah et al., 2006). Although the antibacterial properties of EOs have been long recognized, the recent interest in alternative naturally derived antimicrobials has lead to a renewed scientific interest in these substances. Many in vitro studies report a high efficacy of EOs against food-borne pathogens and spoilage bacteria (Smith-Palmer et al., 1998; Hammer et al., 1999; Elgayyar et al., 2001; Dorman and Deans, 2002). However, a higher concentration of EO is needed to achieve the same effect in food as *in vitro* (Burt, 2004). If EOs are expected to be widely applied as antibacterials, the organoleptical impact should be considered as the use of naturally derived preservatives can alter the taste of food or exceed acceptable flavour thresholds (Hsieh et al., 2001; Nazer et al., 2005). Thus, combinations of plant extracts may help to minimise concentrations and consequently reduce sensory impact. Furthermore, these combinations may also control some bacteria that are known to show consistently high resistance to plant antimicrobials, such as *Pseudomonas* spp. (Hammer et al., 1999; Holley and Patel, 2005). Although some studies have concluded that whole EOs have a greater antibacterial activity than the major components mixed (Gill et al., 2002; Mourey and Canillac, 2002), the combination of these major components with other components that have a weaker activity can achieve a synergistic effect (Ultee et al., 2000).

In general, the efficacy of many added and naturally occurring antimicrobials may be reduced by certain food components (Glass and Johnson, 2004). It is supposed that high levels of fat and/or protein in foodstuffs protect bacteria from the action of EOs (Aureli et al., 1992; Pandit and Shelef, 1994; Tassou al., 1995). Carbohydrates in foods do not appear to protect bacteria from the antimicrobial effects of EOs (Shelef et al., 1984). Gill et al. (2002) suggested that the greater availability of nutrients in foods compared to laboratory media may enable bacteria to repair damaged cells faster. In this respect not only the intrinsic properties of the food are important but extrinsic determinants, such as temperature or characteristics of bacteria, can affect bacterial sensitivity (Burt, 2004). Since most foods are mainly composed of water, carbohydrates, fats, proteins and NaCl, it is important to analyse the influence of these components on the antimicrobial activity of any proposed antimicrobial compound (Devlieghere et al., 2004).

Recent studies have shown that plant extracts are useful for reduction of pathogens associated with chicken frankfurters (Mytle et al., 2006) or cooked beef (Ahn et al., 2007). On the contrary, some authors recorded very low antimicrobial activity or no effect against *E. coli* O157:H7 or *Salmonella* when EOs were applied to ground beef (Uhart et al., 2005) or ready-to-cook chicken (Firouzi et al., (2007), respectively. The application of plant EOs for control of foodborne pathogens and food spoilage bacteria requires the evaluation of a number of aspects; the effects on organoleptic properties, evaluation of the range of activity against the organisms of concern to a particular product as well as food compositional effects on activity. Optimal application in real food systems depends on these factors, therefore, the application of EOs in food should incorporate studies to determine and quantify the effect of food ingredients on their antimicrobial activity. This work aims to bridge *in vitro* studies with practical application of EOs through assessing the effects of a range of concentrations of main food components based on meat and vegetable substrates prior to application in complex food systems. Thus, the objectives of this study were to evaluate and quantify the effect of a range of plant EOs in combination, against four pathogens, *B. cereus*, *E. coli*, *L. monocytogenes* and *P. aeruginosa*, and to determine interactive effects with protein, carbohydrate, oil and pH levels in order to optimise applications in food.

2. Material and methods

2.1. Essential oils

The essential oils (EOs) selected for this study and their composition are indicated in Table 1. The selection was based on a balance between reported antimicrobial efficacy, sensory properties and presence of different components in the EOs. The EOs were obtained from Guinness Chemical Ltd. (Portlaoise, Ireland) and were CO_2 soluble SE-extracts from leaves.

2.2. Bacteria

The bacteria used in this study were *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* IL323 and *Pseudomonas aeruginosa* ATCC 27853. The product isolated *Listeria* strain was kindly provided by the Department of Life Sciences, University of Limerick, Ireland. All cultures were maintained at -70ºC in 20% glycerol and grown in Tryptic Soy Broth (TSB, pH 7.2, Scharlau Chemie) at 37ºC for 24 hours to obtain sub-cultures. Working cultures were prepared from sub-cultures and grown under optimal conditions for each bacterium for 18 hours. Working cultures were adjusted to the required concentration of 10^6 CFU/ml using the McFarland standard (Biomerieux Inc.).

2.3. Synergy studies

Combinations of EOs were qualitatively assessed using the spot-on-agar test. The effect of varying concentrations on efficacy of EO combinations was determined using *E. coli* ATCC 25922 as a test organism. Fractional inhibitory concentration (FIC) indices were calculated using the checkerboard method to quantify the potential synergy of oregano, marjoram or thyme in combination with the remainder of selected EOs. Assays were performed in duplicate and then replicated.

2.3.1. Spot-on-agar test

The spot on agar test was performed based on previous work (Cintas et al., 1998) but with modifications. Ten µl of EO extracts diluted in ethanol at 10% (v/v) were spotted onto TSA (1.2%) plates seeded with 10^6 CFU/ml of the indicator strain. Combinations were initially assessed in a 1:1 ratio. Spotted plates were then incubated at 37°C for 18 h. Ethanol was used as control and inhibition zones around the wells were measured in millimetres.

2.3.2. Effect of varying concentrations on efficacy of EO combinations against E. coli

The influence of varying concentration of EO combinations on efficacy was assessed against *E. coli* using 96-well micro titer plates (Sarstedt Ltd). The first row of each plate contained 100 µl of the EO extract or the EO combination (1:1) diluted in TSB. The range of initial concentrations for each of the EOs is indicated in Table 2. Wells containing EOs in the first row were then diluted two fold along each column. At least 2 columns were used for controls. Positive controls contained growth media inoculated with the organism under investigation. Negative controls contained EOs and sterile growth media only. Finally, 100 μl of media containing $2x10^6$ CFU/ml of the bacterium were added to all wells. The plates were then placed in a microplate spectrophotometer (PowerWave, Biotek) set at 37°C. The absorbance readings were taken at 600 nm every 30 minutes for an 18 hours incubation period and kinetic curves were analysed.

2.3.3. Checkerboard Method

The checkerboard method was performed using 96-well microtitre plates as described previously (Moody, 2003; Schelz et al., 2006), to obtain the FIC index. The microplate assay was arranged as follows: EO_A was diluted two-fold along the x-axis, whilst EO_B was diluted two-fold along the y-axis. The final volume in each well was 100 μl comprising 50 μl of each EO dilution. Subsequently, 100 μl of media containing $2x10^6$ CFU/ml of the indicator strain were added to all wells. The plates were then incubated at 37 °C for 18 h. The FIC indices were calculated as $FIC_A + FIC_B$, where FIC_A and FIC_B are the minimum concentrations that inhibited the bacterial growth for EOs A and B, respectively. Thus, FICs were calculated as follows: FIC_A = (MIC_A combination / MIC_A alone) and FIC_B = (MIC_B combination / MIC_B alone). The results were interpreted as synergy (FIC < 0.5), addition (0.5 ≤ FIC ≤ 1), indifference (1 < FIC ≤ 4) or antagonism (FIC > 4). The concentrations used for oregano, marjoram and thyme, either alone or in combination with the remainder of the EOs, are shown in Table 3.

2.4. Interactive effects of food ingredients and pH

2.4.1. Food ingredients and pH assays

The effect of food ingredients and pH on the antimicrobial efficacy of EOs was performed using a range of model media and *Listeria monocytogenes* IL323 as indicator strain. The plant EOs were fixed factors: oregano (30 ppm) and thyme (60 ppm) were independently assessed. Model media were comprised of the following: (i) water soluble starch from potato (0, 1, 5 or 10%, Sigma-Aldrich Ireland Ltd) in TSB; (ii) beef extract (1.5, 3, 6 or 12%, Scharlau Chemie) in deionized water; and (iii) sunflower oil (0, 1, 5 or 10%) in TSB. Model media containing starch or beef extract were autoclaved prior to use. For the oil model media, the sunflower oil was autoclaved separately and then added to sterile TSB. Filter-sterilized Tween 80 (Merck) was added at 0.1% to facilitate mixing and to stabilize the emulsion. The pH of each model media was adjusted to 7.2. To determine the effect of pH on EO efficacy TSB was adjusted to pH 4, 5, 6 or 7 with 1 N HCl solution.

The growth of *L. monocytogenes* in each model medium with oregano or thyme was monitored using 96 well-microplates. 200 µl of each medium containing 2×10^6 CFU/ml of the *Listeria* strain were added to wells of 96 well-microplates, which were assessed in microplate spectrophotometer as described above. Positive controls contained model media inoculated with the organism under investigation. Negative controls contained EOs and sterile model media only. The survival curves of *Listeria monocytogenes* IL323 in model media were monitored at 600 nm over a 24 hour period.

2.5 Kinetic analysis

The kinetic curves were analyzed by the KC4 software (Biotek) calculating the increase in lag phase (λ) and the maximum specific growth rate (μmax). Statistical analysis on data was performed using SPSS 15.0 (SPSS Inc., Chicago, U.S.A). Data represents the means of experiments performed in duplicate and replicated at least twice. Means were compared using ANOVA followed by LSD testing at $p < 0.05$ level.

3. Results

3.1. Synergy studies

3.1.1. Spot on agar test and effect of varying concentrations on efficacy of EO combinations

All the EO combinations were evaluated by the spot-on-agar-test. Only oregano in combination with thyme showed a greater efficacy than when assessed individually (results not shown). EO mixtures were also assessed monitoring the effect of varying concentrations on *E. coli* (Table 2). Some combinations resulted in lag phase extensions and reductions in the μmax at half the concentrations of the individual EOs. The lag phase duration of *E. coli*, when exposed to oregano in combination with basil, was significantly increased by 7.44h with respect to the increase recorded by oregano alone ($p < 0.05$). The combination of oregano with lemon balm resulted in a 3.55h extension of the lag phase by comparison with lemon balm alone. When oregano was combined with marjoram, the reduction in the maximum specific growth rate achieved was approximately 3 fold higher than that with the EOs alone ($p < 0.05$). Combining oregano with sage or thyme increased the lag phase of *E. coli*, by comparison with the individual

EOs, but when oregano was combined with rosemary, there was no benefit compared to the EOs alone.

3.1.2. Checkerboard method

The quantitative effects of oregano, marjoram or thyme in combination with the other EOs are described in terms of FIC indices. The FIC's of oregano in combination with the other EOs are shown in Table 4. None of these combinations displayed a synergistic activity against the bacteria used in this study. However, oregano combined with all the other EOs had additive effects against *B. cereus*, but no additive or synergistic effects were recorded for oregano based combinations against *L. monocytogenes*. The combinations of oregano with marjoram and oregano with thyme had additive effects against *E. coli* and *P. aeruginosa*, respectively, while oregano in combination with basil had a useful additive activity against both Gram-negative organisms.

The FIC's for both marjoram and thyme combinations against *L. monocytogenes* are shown in Table 5. A similar trend was noted for both EOs, where additive effects were observed in combinations with basil, rosemary and sage.

3.2. Interaction with food ingredients and pH

The lag phase (λ) and the maximum specific growth rate (μmax) of *L. monocytogenes* IL323 grown in different model media are indicated in Tables 6 and 7, respectively.

3.2.1. Effect of protein

To investigate the effect of proteins on the antimicrobial activity of oregano and thyme, growth experiments with *L. monocytogenes* were performed in beef extract at 4 different concentrations (1.5, 3, 6, and 12%). The lag phase of *L. monocytogenes* grown in beef extract containing oregano was longer than the control at protein concentrations of 6% and 12% ($p < 0.05$). The antimicrobial activity of thyme was increased in high protein concentrations, leading to a significantly longer lag phase from 3% to 12% of protein, with respect to the control ($p < 0.05$). In the control culture, a longer lag phase and a lower growth rate were observed at protein concentrations of 1.5% and 3% ($p < 0.05$). The growth rate of *Listeria* grown in beef extract with thyme was higher than the control at protein concentrations of 3% and 6% ($p < 0.05$).

3.2.2. Effect of starch

Four different concentrations of potato starch (0, 1, 5 and 10%) were tested to determine the influence of carbohydrates on the efficacy of oregano and thyme. The lag phase of *L. monocytogenes* grown in starch model media containing oregano or thyme decreased in either 5% or 10% starch concentration. Low concentrations of this carbohydrate had a positive influence on the EO antimicrobial activity, with higher lag phases by comparison with the control ($p < 0.05$). In general, the growth rate of *L. monocytogenes* decreased at higher starch concentrations. There was no significant difference in the growth rate of *Listeria* within starch model media at any concentration whether it contained EOs or not ($p < 0.05$).

3.2.3. Effect of sunflower oil

To assess the effect of oil on the EO antimicrobial activity, the growth of *L. monocytogenes* was monitored in model media containing sunflower oil concentrations of 0, 1, 5 and 10%. When EOs were included in the oil media, *L. monocytogenes* had a shorter lag phase at higher oil concentrations ($p < 0.05$). The growth rate decreased at 10% oil. The addition of EOs to the varying concentrations of oil media did not significantly effect the growth rate by comparison with controls.

3.2.4. Effect of pH

The effect of pH on the EO antimicrobial activity was evaluated using TSB at pH 4, 5, 6 and 7 (Tables 6, 7). *L. monocytogenes* did not grow at pH 4. The lag phase of *Listeria* grown in pH model media with EO was longer than that recorded in EO free controls ($p < 0.05$), especially at pH 5, however, the lag phase was greatest at pH 5 in control media also. The growth rate of *L. monocytogenes* increased at higher pH values, regardless of presence or absence of EOs.

4. Discussion

Since higher concentrations of plant EOs are generally required when added to food, the application of EOs in food may be limited due to changes in organoleptic and textural quality of food or interactions of EOs with food components (Devlieghere et al., 2004). Accordingly, a challenge for practical application of EOs is to develop optimised low dose combinations to maintain product safety and shelf-life, thereby minimising the undesirable flavour and sensory changes associated with the addition of high concentrations of EOs.

In this work, combinations of selected EOs (basil, lemon balm, marjoram, oregano, rosemary, sage and thyme) were tested against *B. cereus*, *E. coli*, *L. monocytogenes* and *P. aeruginosa*. Preliminary studies showed that the combination of oregano with thyme had a greater efficacy than the EOs separately against the four pathogens. Furthermore, the combinations of oregano with lemon balm and basil increased the lag phase of *E. coli* by 3.55h and 7.44h, respectively, by comparison with oregano alone. When oregano was combined with marjoram, the maximum specific growth rate was reduced 3 times, with respect to both EOs individually. There were important additive effects found when FIC calculations from the extended study of oregano, marjoram and thyme in combination with the remainder of the EOs were performed. Although none of the combinations showed clear synergistic effects, combining EOs selected in this study, at lower concentrations than required for the EOs alone, has potential for practical application to extend the shelf-life of selected foods.

Burt (2004) suggested that the minor components present in the EOs extracts are more critical to the activity than EOs main components mixed, and may have a synergistic effect or potentiating influence. In many cases the result was an "additive effect". All the oregano combinations were additive against *B. cereus*. The following EO combinations also showed additive effects: oregano in combination with basil or thyme against *E. coli* and *P. aeruginosa*, oregano combined with marjoram against *E. coli*, and marjoram and thyme mixed with basil, rosemary or sage against *L. monocytogenes*. These results can be explained considering the efficacy of the main components of EOs individually. In general, EOs possessing the strongest antibacterial properties contain a high percentage of carvacrol and/or thymol, such as oregano or thyme (Elggayar et al., 2001; Dorman and Deans, 2002; Burt, 2004; Oussalah et al., 2006). Since hydroxyl groups and allylic side chains enhance EO efficacy (Burt, 2004), linalool and 4-thujanol, which are the main components of basil and marjoram, respectively, may have contributed to the promising antimicrobial activity achieved with their combinations. Previous studies reported the high antimicrobial activity of linalool or basil against Gram-negative bacteria (Elggayar et al., 2001; Oussalah et al., 2006). Acetate moieties in EO compounds may also positively influence the activity of marjoram (Dorman and Deans, 2000). The main components of rosemary and sage, which are camphor and eucalyptol, possess oxygen functions in their structure and these functions are known to increase the antimicrobial properties of terpenoids (Naigre et al., 1996) A major component of sage, β-caryophyllene, also has a high antimicrobial activity against Gram-positive bacteria (Longaray Delamare et al., 2005). As antimicrobial activity depends not only on chemical composition but also on lipophilic properties, the potency of functional groups or aqueous solubility (Dorman and Deans, 2000) and the mixture of compounds with different biochemical properties may increase EO efficacy.

However, the mechanism of action as well as EO composition deserves to be studied in more detail in order to elucidate why combinations of EOs with a strong individual antimicrobial efficacy such as oregano and thyme, did not show synergistic effects, and why on the other hand combinations of two EOs with individually moderate activity resulted in enhanced effects in combination, such as marjoram, basil, rosemary or sage. Lambert et al., (2001), reported that carvacrol and thymol in combination were additive against *S. aureus* and *P. aeruginosa*. Nazer et al., (2004), found that thymol in combination with other aromatic compounds led to improved inhibition, but no real synergistic effect was demonstrated between compounds against *Salmonella*. As plant EOs possess similar composition, their combinations may exhibit addition rather than a synergistic effect. As a result, combinations with other compounds containing different chemical structures may improve the EO efficacy. For example, synergism between carvacrol and its precursor *p-cymene* has been noted (Ultee et al., 2000). It appears that *p-cymene*, a very weak antibacterial, swells bacterial cell membranes to a greater extent than carvacrol does, so *p-cymene* probably enables carvacrol to be more easily transported into the cell. Lin *et al*., (2004) reported a 1 Log cycle reduction of *L. monocytogenes* using an oregano and cranberry EO combination (ratio, 75:25) and although the reduction was 6 $\log_{10}$CFU higher with the addition of lactic acid, the authors suggested that synergistic effects did not occur with the EOs tested. Thus, EOs may be employed in combination with other food preservation hurdles in order to control the growth of pathogens and spoilage in food at low doses. In this context, some authors proposed that the combination of essential oil constituents with other natural preservatives, such as bacteriocins or fatty acids, promoted their efficiency against food-borne pathogens (Yamazaki et al., 2004; Grande et al., 2007).

Recently, some studies have shown successful or potential applications of EOs and their compounds, alone or in combination with other preservation methods, in order to reduce or control the presence of foodborne pathogens and spoilage microorganisms on food produce, such as fruit (Raybaudi-Massilia et al., 2006; Martinez-Romero et al., 2007), fish (Mahmoud et al., 2006), meat (Mytle et al., 2006; Ahn et al., 2007; Ghalfi et al., 2007; Solomakos et al., 2008), or milk (Cava et al., 2007). However, in some cases the EO activity decreased considerably when added to a complex food system. For example, Firouzi et al., (2007) found that oregano and nutmeg were effective against *E. coli* O157:H7 in a broth system, but had no effect in ready-to-cook chicken. Uhart et al., (2005), also observed that when in direct contact, spices inactivated *S. typhimurium* DT104, but that the activity decreased when applied to ground beef. Thus, an important aspect for the optimised application of plant EOs is the evaluation of efficacy within food products or in model systems that closely simulate food composition, prior to application on real food. In this study four different model media were prepared to assess and quantify the effect of food components on the antimicrobial efficacy of oregano and thyme against *L. monocytogenes*.

The presence of high concentrations of protein promoted the growth of *L. monocytogenes*, however the efficacy of oregano and thyme was also greater at these higher concentrations of protein. The beef extract culture medium was constituted mainly of peptones, which may have displayed hydrophobic properties with consequent interactions with EOs to facilitate their dissolution in this medium. Baranauskien et al., (2006), reported that proteins usually possess a high binding capacity for flavor volatile compounds. However, other studies have shown that milk proteins are limiting factors for antimicrobial efficacy (Pol et al., 2001, Smith-Palmer et al., 2001, Devlieghere et al., 2004).

The antimicrobial activity of EOs was very high at pH 5. Previously, it was also observed that the inhibitory effect of plant extracts was greater at acidic pH values (Del Campo et al., 2000; Hsieh et al., 2001). The susceptibility of bacteria to EOs appears to increase with lower pH values since the hydrophobicity of EOs increases at low pH, consequently enabling easier dissolution in the lipids of the cell membrane of target bacteria (Juven et al., 1994). The major efficacy of EOs at pH 5 was confirmed with the lag phase and growth rate results for *Listeria* at this pH, which were longer and lower, respectively, than at pH 6 or 7. Similar trends with regard to pH effects were observed within control media without EOs to those containing EOs. As the pH was reduced, the lag phase increased and the growth rate declined for all tests. Furthermore, the addition of either Oregano or Thyme, particularly to media at pH 5, enhanced the reduction of the growth rate and the extension of the lag phase.

High concentrations of sunflower oil had a negative influence on the antimicrobial activity of oregano and thyme EOs. Singh *et al*. (2003) reported that thyme EO reduced bacterial populations significantly in zero- and low-fat hotdogs, but not in full-fat hotdogs. Cava et al. (2007) found that the antimicrobial activity of cinnamon and clove EOs against *L. monocytogenes* was reduced in milk samples with higher fat content. Similarly, Smith-Palmer et al. (2001) observed that EOs were less effective in full-fat soft cheese than in low-fat soft cheese. Canillac and Mourey (2004) also observed that the addition of dairy fat into a test medium reduced the antilisterial efficacy of *Picea excelsa* EO. Previously, mint oil was found to exhibit little antibacterial effect against *L. monocytogenes* and *S. enteritidis* in high fat products, whereas in low fat food the same EO was much more effective (Tassou *et al*., 1995). In this context, Glass and Johnson (2004) reported that the antibotulinal effects of nisin and fatty acids were reduced by 20% milk fat or soybean oil. Mejlholm and Dalgaard (2002) suggested that if the EOs generally dissolve in the lipid phase there will be relatively less available to act on bacteria present in the aqueous phase.

The EO efficacy was also reduced at high concentrations of starch, in contrast to the general observation that carbohydrates in foods do not protect bacteria from the action of EOs as much as fat and protein do (Shelef et al., 1984). Devlieghere et al. (2004) reported a protective effect of carbohydrate for bacteria where starch at 30% had a negative impact on the antimicrobial activity of chitosan. Ofman et al. (2004), also observed a negative effect of tapioca starch on antimicrobial activity of preservatives.

Control of *L. monocytogenes* contamination, survival or growth in ready-to-eat (RTE) meat is a major challenge confronting the food industry (Singh et al., 2003; Mytle et al., 2006; Busatta et al., 2008). Organic acids, steam and hot water treatments are commonly used as decontamination treatments, but *L. monocytogenes* can thrive in the environment of meat processing facilities. Singh et al. (2003) suggested that the use of EOs in conjunction with other preservation techniques such as chemical preservatives or low temperature could develop a synergistic alternative to current methods. Based on the results shown in this work, EO mixtures may be suitable for application within surface decontamination protocols or inclusion as ingredients in ready to use raw or cooked foods. This work shows a link between food composition and EO antimicrobial efficacy, therefore careful selection of EO combinations taking food composition into account prior to application development is important. Low dose combinations of EOs may be useful for control of food safety in low fat RTE-meat based products where the sensory characteristics could be desirable or in lower pH foods where a risk of pathogen survival exists. Incorporation of organic acids may also enhance the efficacy of EO combinations for preservation of quality and safety in real food systems for extended periods.

5. Conclusions

The antimicrobial efficacy of the EOs in this study was found to be a function of ingredient manipulation. The antimicrobial activity of oregano and thyme against *L. monocytogenes* was increased at higher protein concentrations and moderately acidic pH conditions. Concentrations above 5% of potato starch or sunflower oil reduced EO efficacy. Therefore, the application of EOs should be further investigated for control of microbial safety and spoilage concerns in proteinaceous foods and/or foods with low pH values, which may promote the antibacterial efficacy of EOs. The retention of anti-microbial efficacy of EOs within suitable food systems should be evaluated alone as well as taking hurdle effects of other preservation methods into account.

Combinations of plant EOs were assessed for synergistic activity, as this would allow lower concentrations of EOs to be used, thereby achieving the twin aims of reducing any undesirable organoleptic impact, as well as controlling food-borne pathogens and spoilage bacteria in food. No synergy was found but addition occurred with a number of combinations. Oregano and thyme were the most effective EOs applied individually, and in combination they produced an additive effect against *B. cereus* and *P. aeruginosa*. The combinations of oregano with marjoram and thyme with sage had promising efficacy against *E. coli* and *L. monocytogenes* respectively. Thus, oregano combined with thyme at low doses should be considered as a potential alternative for control of pathogens as well as microbial spoilage issues, while the combinations of oregano with marjoram or thyme with sage might be useful for targeted control of key Gram-negative or Gram-positive bacteria, respectively.

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Table 1

Essential oils selected for this study

Essential oil	Scientific name	Main components (%)
Basil	*Ocimum basilicum*	Linalool (42.3) Estragole (26.9) Eucalyptol (8.1)
Lemon balm	*Melissa officinalis*	Citral (Neral/Geranial) (22.4/36.7) Caryophyllene (13.2)
Marjoram	*Origanum majorana*	4-Thujanol (36.2) Sabinene hydrate acetate (16.8) Terpinene-4-ol (8.7)
Oregano	*Origanum vulgare*	Carvacrol (68.5) Thymoquinone (12.1) p-Cymene (7.8)
Rosemary	*Rosmarinus officinalis*	Eucalyptol (39.6) Camphor (19) Alpha Pinene (4.8)
Sage	*Salvia triloba*	Eucalyptol (42.0) Camphor (12.0) Caryophyllene (7.2)
Thyme	*Thymus vulgaris*	Thymol (52.9) p-Cymene (34.0)

Table 2

Effect of EO combinations on growth parameters of *E. coli* ATCC 25922

EOs (Individual)		**Lag phase**[a]		**Maximum specific growth rate**[b]	
			St. Dev.		St. Dev.
Oregano	300 ppm	3.84	± 0.21	0.367	± 0.042
Basil	10,000 ppm	4.86	± 0.30	0.452	± 0.005
Lemon balm	20,000 ppm	5.21	± 0.43	0.401	± 0.019
Marjoram	2,000 ppm	3.77	± 1.01	0.406	± 0.021
Rosemary	10,000 ppm	4.55	± 0.70	0.480	± 0.196
Sage	50,000 ppm	4.80	± 1.61	0.432	± 0.016
Thyme	600 ppm	3.29	± 0.07	0.400	± 0.006
EO combinations[c]					
Oregano + Basil	(1/2)	11.28	± 3.05	0.452	± 0.004
Oregano + Basil	(1/4)	4.21	± 0.19	0.449	± 0.004
Oregano + Lemon balm	(1/2)	8.76	± 0.18	0.312	± 0.023
Oregano + Lemon balm	(1/4)	5.69	± 0.18	0.407	± 0.015
Oregano + Marjoram	(1/2)	4.05	± 1.23	0.117	± 0.043
Oregano + Marjoram	(1/4)	2.86	± 0.34	0.404	± 0.032
Oregano + Rosemary	(1/2)	5.84	± 1.37	0.383	± 0.018
Oregano + Rosemary	(1/4)	3.76	± 0.81	0.420	± 0.006
Oregano + Sage	(1/2)	8.50	± 1.25	0.419	± 0.019
Oregano + Sage	(1/4)	4.67	± 0.51	0.425	± 0.005
Oregano + Thyme	(1/2)	6.32	± 0.57	0.376	± 0.025
Oregano + Thyme	(1/4)	3.08	± 0.17	0.395	± 0.009

[a] Lag phase is expressed in hours.

[b] Maximum specific growth rate is expressed in hours^{-1}.

[c] EO combinations were assessed at 50% (1/2) and 25% (1/4) of the individual concentrations.

Table 3

Concentrations[a] of EOs used alone (A) or in combination (C) against the bacteria selected for this study

EOs	*B. cereus* ATCC 11778		*E. coli* ATCC 25922		*L. monocytogenes* IL323		*P. aeruginosa* ATCC 27853	
	A	C	A	C	A	C	A	C
Basil	20,000	10,000	20,000	10,000	20,000	10,000	200,000	100,000
Lemon balm	50,000	25,000	40,000	20,000	2,000	1,000	200,000	100,000
Marjoram	10,000	5,000	4,000	2,000	8,000	4,000	100,000	50,000
Oregano	500	250	400	200	200	100	4,000	2,000
Rosemary	20,000	10,000	20,000	10,000	10,000	5,000	200,000	100,000
Sage	200,000	100,000	200,000	100,000	5,000	2,500	200,000	100,000
Thyme	1,000	500	1,000	500	200	100	5,000	2,500

[a] Concentrations are expressed in ppm

Table 4

FIC indices of oregano combinations

Combinations	***B. cereus* ATCC 11778**		***E. coli* ATCC 25922**		***L. monocytogenes* IL323**		***P. aeruginosa* ATCC 27853**	
	FIC	Stdev[b]	FIC	Stdev[b]	FIC	Stdev[b]	FIC	Stdev[b]
Oregano +								
Basil	0.83 (A)[a]	± 0.14	1.00 (A)	± 0.00	1.25 (I)	± 0.35	1.00 (A)	± 0.00
Lemon balm	0.86 (A)	± 0.18	1.17 (I)	± 0.34	1.25 (I)	± 0.43	1.38 (I)	± 0.12
Marjoram	0.75 (A)	± 0.15	0.83 (A)	± 0.30	1.18 (I)	± 0.40	1.75 (I)	± 0.35
Rosemary	0.79 (A)	± 0.20	1.83 (I)	± 0.29	1.50 (I)	± 0.00	1.50 (I)	± 0.00
Sage	1.00 (A)	± 0.00	1.38 (I)	± 0.18	1.75 (I)	± 0.35	1.50 (I)	± 0.00
Thyme	0.78 (A)	± 0.16	1.17 (I)	± 0.30	1.18 (I)	± 0.30	0.88 (A)	± 0.18

[a] Results are interpreted as synergy (**S**, FIC < 0.5), addition (**A**, 0.5 ≤ FIC ≤ 1), indifference (**I**, 1 < FIC ≤ 4) or antagonism (**AN**, FIC > 4).

[b] Standard deviation

Table 5

FIC indices of marjoram and thyme combinations against *L. monocytogenes* IL323

Combinations	**Marjoram**		**Thyme**	
	FIC	Stdev[b]	FIC	Stdev[b]
Marjoram or thyme +				
Basil	0.75 (A)[a]	± 0.51	0.94 (A)	± 0.44
Lemon balm	1.25 (I)	± 0.00	1.25 (I)	± 0.35
Marjoram	-		1.55 (I)	± 0.57
Oregano	1.18 (I)	± 0.40	1.18 (I)	± 0.30
Rosemary	1.03 (A)	± 0.54	1.06 (A)	± 0.62
Sage	1.00 (A)	± 0.25	1.00 (A)	± 0.00
Thyme	1.55 (I)	± 0.57	-	

[a] Results are interpreted as synergy (**S**, FIC < 0.5), addition (**A**, 0.5 ≤ FIC ≤ 1), indifference (**I**, 1 < FIC ≤ 4) or antagonism (**AN**, FIC > 4).

[b] Standard deviation

Table 6

Lag phase (λ) of *L. monocytogenes* IL323 grown in model media containing oregano (30 ppm) or thyme (60 ppm)

Model media	Oregano		Thyme		Control[c]	
	λ (h)[a]	Stdev[b]	λ (h)[a]	Stdev	λ (h)[a]	Stdev
Beef extract						
1.5 %	7.39	± 0.96	9.15	± 2.57	8.01	± 2.63
3.0 %	7.81	± 1.22	11.79	± 1.33	6.14	± 0.08
6.0 %	10.75	± 2.31	10.94	± 2.51	6.48	± 0.20
12.0 %	10.79	± 1.53	9.75	± 2.16	6.33	± 0.37
Starch media						
0.0%	15.01	± 1.40	12.06	± 2.70	7.80	± 0.93
1.0%	13.96	± 3.66	11.69	± 2.08	7.04	± 0.17
5.0%	9.71	± 0.89	8.87	± 3.56	7.58	± 0.30
10.0%	8.84	± 1.52	8.43	± 2.38	8.04	± 0.75
Sunflower oil media						
0.0%	15.23	± 0.05	15.05	± 0.45	7.75	±0.13
1.0%	14.21	± 0.33	12.54	± 0.06	7.31	±0.10
5.0%	10.50	± 0.13	9.22	± 0.30	7.24	±0.10
10.0%	9.17	± 0.14	9.47	± 0.03	7.33	±0.18
pH						
TSB pH 4	0.00	± 0.00	0.00	± 0.00	0.00	± 0.00
TSB pH 5	12.43	± 0.44	16.13	± 1.28	9.55	± 1.83
TSB pH 6	7.70	± 0.73	9.48	± 1.51	6.75	± 1.57
TSB pH 7	9.50	± 0.09	10.80	± 0.95	6.88	± 1.10

[a] Lag phase is expressed in hours.

[b] Standard deviations are indicated beside each value.

[c] *L. monocytogenes* grown in model media without any EO was used as the control.

Table 7

Maximum specific growth rate (μmax) of *L. monocytogenes* IL323 grown in model media containing oregano (30 ppm) or thyme (60 ppm)

Model media	Oregano		Thyme		Control[c]	
	μmax (h^{-1})[a]	Stdev[b]	μmax (h^{-1})[a]	Stdev	μmax (h^{-1})[a]	Stdev
Beef extract						
1.5 %	0.034	± 0.006	0.079	± 0.022	0.054	± 0.037
3.0 %	0.076	± 0.041	0.146	± 0.077	0.074	± 0.013
6.0 %	0.099	± 0.037	0.195	± 0.068	0.117	± 0.032
12.0 %	0.195	± 0.018	0.200	± 0.019	0.215	± 0.001
Starch media						
0.0%	0.185	± 0.058	0.250	± 0.021	0.238	± 0.040
1.0%	0.208	± 0.082	0.271	± 0.014	0.268	± 0.058
5.0%	0.142	± 0.032	0.164	± 0.020	0.147	± 0.008
10.0%	0.098	± 0.016	0.104	± 0.003	0.097	± 0.002
Sunflower oil media						
0.0%	0.240	± 0.015	0.231	± 0.008	0.279	± 0.008
1.0%	0.226	± 0.001	0.209	± 0.004	0.223	± 0.007
5.0%	0.208	± 0.012	0.235	± 0.005	0.200	± 0.018
10.0%	0.174	± 0.013	0.220	± 0.004	0.170	± 0.014
pH						
TSB pH 4	0.000	± 0.000	0.000	± 0.000	0.000	± 0.000
TSB pH 5	0.004	± 0.001	0.016	± 0.006	0.017	± 0.018
TSB pH 6	0.141	± 0.020	0.175	± 0.025	0.173	± 0.064
TSB pH 7	0.284	± 0.022	0.328	± 0.011	0.261	± 0.054

[a] Maximum specific growth rate is expressed in $hours^{-1}$.

[b] Standard deviations are indicated beside each value.

[c] *L. monocytogenes* grown in model media without any EO was used as the control.